Quantification of chlorination by-products of BPA

To identify the reaction products of BPA by chlorination, BPA solution was reacted with chlorine for a few minutes and analyzed by LC/MS as preliminary study. As a result, BPA (m/z 227) was observed on the retention time at 5.18 min, and several chlorinated BPAs were typically detected. The quasi-molecular ion at m/z 261 (One chlorine atom substituted products of BPA) was observed at 6.67 min, and m/z 295 (two chlorine atoms), m/z 331 (three chlorine atoms) and m/z 365 (four chlorine atoms) were observed at 7.39, 9.17 and 11.07 min, respectively. Further analysis was conducted about the change in concentration of four chlorinated BPAs with the chlorination time. Fig.3 shows the change in concentration of BPA and four chlorinated BPAs with chlorination time. The concentration of BPA was 30 μg/L after 60 min chlorination. This result indicated that more than 85 % of BPA had disappeared after 60 min reaction time. Four chlorinated BPAs were formed from the beginning of chlorination. Maximum concentrations of Cl-BPA (50 μg/L), diCl-BPA (35 μg/L), triCl-BPA (18 μg/L) and tetraCl-BPA (16 μg/L) were observed after 5 min, 45 min, 45 min and 90 min, respectively. Such chlorination by-products decreased with the increase in reaction time in the presence of free chlorine.

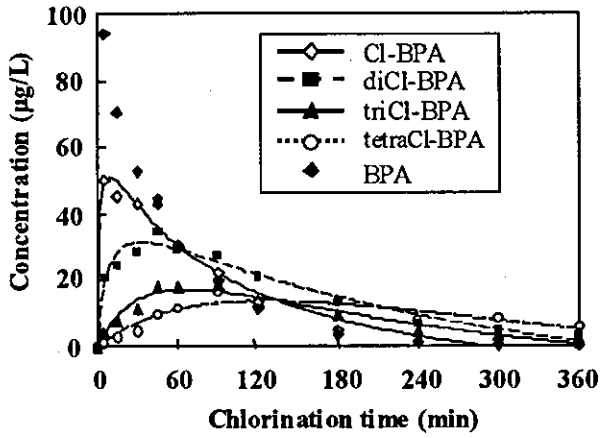

Fig.3 Effects of chlorination time on the concentration of chlorinated BPAs and BPA

Contribution of chlorinated BPAs to the total estrogenic activities

To evaluate the contribution of chlorinated BPAs to the total estrogenic activities, fractionation of chlorinated BPAs were conducted by use of LC. The time series data of estrogenic activity at c.f.10000 and the concentration of fractionated BPA, Cl-BPA, diCl-BPA, triCl-BPA and tetraCl-BPA are presented in Fig.4a-e, respectively. These fractionations were conducted at 45, 180, 360 and 720 min after chlorination. The concentration of BPA itself decreased to 20% of initial concentration (45 μg/L) after 45 min chlorination (Fig.4a). The estrogenic activity induced by BPA slightly decreased at 45min, and was eliminated after 180 min chlorination. The concentration of Cl-BPA was 43 μg/L at 45 min chlorination, and the estrogenic activity of Cl-BPA was higher than initial BPA (Fig.4b). This result indicated that Cl-BPA has stronger estrogenic activity than BPA. However, the estrogenic activity induced by Cl-BPA was eliminated after 360 min chlorination. DiCl-BPA and triCl-BPA induced the estrogenic activities after 45 min chlorination (Fig.4c and 4d). Their estrogenic activities were also eliminated after 720 min. TetraCl-BPA did not induce the estrogenic activity in any chlorination time (Fig.4e). As a result, it is clear that the increase of estrogenic activity after 45 min chlorination at c.f.1000 as shown in Fig.2 was due to the multiple effects of the remaining BPA and newly formed Cl-BPA, diCl-BPA and triCl-BPA. Additionally, the reason for elimination of total estrogenic activity of after 720 min chlorination was due to the

decomposition of these chlorinated BPAs. Although the inhibition of yeast proliferation occurred after 45 min chlorination of BPA at c.f.10000 (data not shown), no such inhibition in yeast proliferation for the fractionated samples was observed. Accordingly, it is suggested that the inhibition of yeast proliferation of the sample after 45 min chlorination of BPA at c.f.10000, should be due to the multiple toxic effects induced by chlorinated BPAs.

The estrogenic activities tested for chlorinated BPAs standards are presented in Fig.5. Apparent decrease of estrogenic activity was observed at higher concentrations of all measured substances. It was not true estrogenic activity because it was due to the toxic effects caused by the inhibition of yeast proliferation. The estrogenic activity of diCl-BPA was the highest of all the measured substances at concentration of 10^{-3} mol/L where it was possible to evaluate the estrogenic activity. The estrogenic activities of Cl-BPA and triCl-BPA were higher than BPA. TetraCl-BPA had little estrogenic activity in the whole concentration ranges.

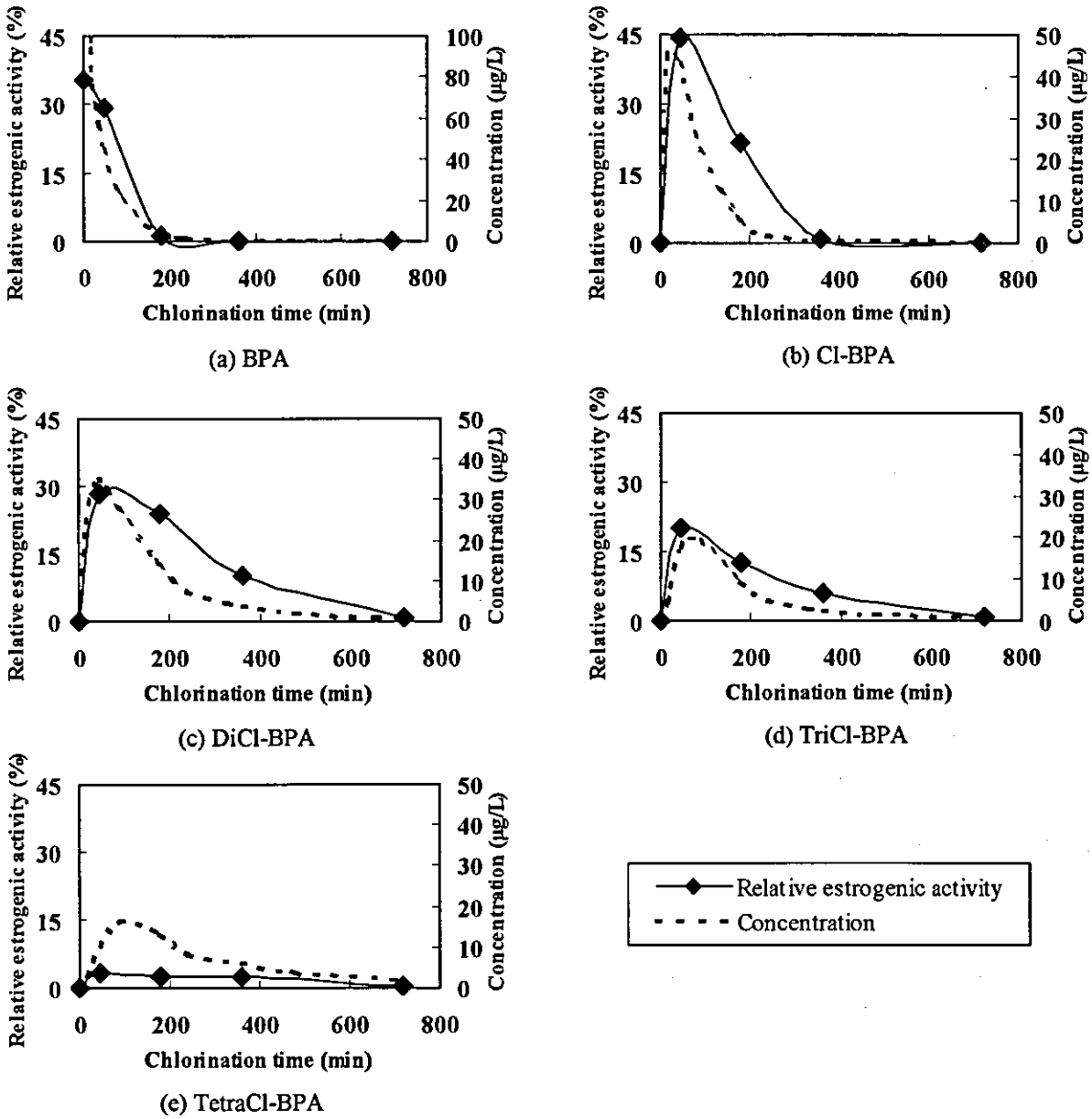

Fig.4 Effect of chlorination time on the formation of chlorination by-products from BPA and their estrogenic activity

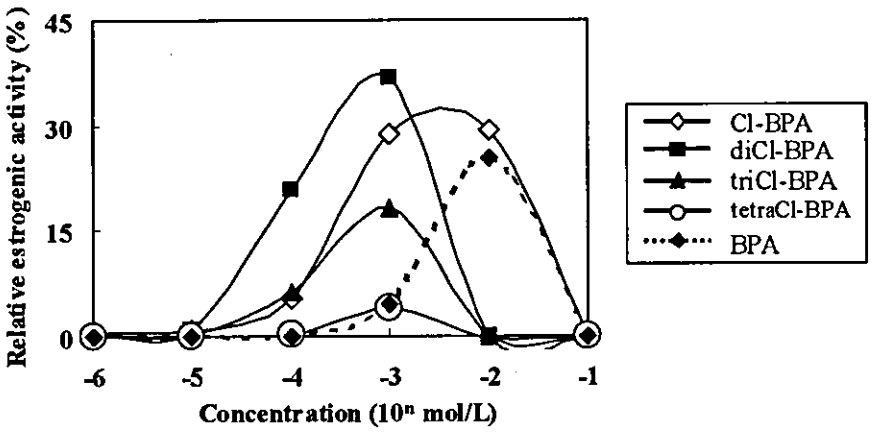

Fig.5 The estrogenic activities induced by the standard solution of chlorinated BPAs and BPA

CONCLUSIONS

The behavior of the estrogenic activity of BPA by chlorination was investigated using yeast two-hybrid assay and LC/MS analysis. Results from this study may be summarized as follows.

- Monochloro BPA, dichloro BPA, trichloro BPA and tetrachloro BPA were typically formed as a result of chlorination of BPA.
- Monochloro BPA, dichloro BPA and tetrachloro BPA induced higher estrogenic activities than BPA itself.
- The formation of monochloro BPA, dichloro BPA and trichloro BPA contributed to the increase of estrogenic activities enhanced in early stage of chlorination.
- The concentrations of these chlorinated BPAs temporarily formed as a result of chlorination with an increase in chlorination time decreased and finally their estrogenic activities were eliminated in the presence of free chlorine.

From these results, it was concluded that chlorination of BPA was effective to eliminate the estrogenic activity of BPA.

ACKNOWLEDGEMENT

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18 三次元蛍光分析を用いた水道水中フミン物質の回収性の検討

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Evaluation of Concentrating Humic Substances in Drinking Water by 3-Dimensional Fluorescence Analysis

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Kyoto University, Takayuki HAYASAKA
Kyoto University, Tomoyuki OKADA

塩素処理後の水道水に含まれるエストロゲン様作用の成分には、塩素処理により作用が増大したものと減少したものがあり、試料調製法に依存して、そのいずれも検出することができる。まず、塩素処理によるエストロゲン様作用増大の要因とされるフミン物質を、OASIS HLB 固相抽出カートリッジを用いて濃縮回収できることを示した。さらに、いくつかのエストロゲン様作用物質に関して回収性の検討を行った。この結果、水道原水および水道水のエストロゲン様作用試験のための試料調製法として、OASIS HLB を用い、水酸化ナトリウムとジクロロメタンの2種類の溶媒を用いて溶出する濃縮法が適当であることを示した。

キーワード：フミン物質、3次元励起・蛍光スペクトル、エストロゲン様作用、塩素消毒
Key words: Humic Substances, 3-Dimensional Excitation-Emission Matrix Spectroscopy, Estrogenic Effect, Chlorination

1. 緒 言

著者らはこれまでに、自然水のエストロゲン様作用とその塩素処理による変化に焦点を当てた検討を行ってきた[1, 2, 3]。結果として、水道水のエストロゲン様作用に関して、水処理の後なお残存する有機物と塩素が反応すればエストロゲン様作用が新たに生成するという点で、トリハロメタン問題と同じ構造を有することを指摘した[2]。

また、自然水中には、フミン物質を中心とする一般有機物と17β-エストラジオールなど個別物質とが含まれるが、塩素処理によってエストロゲン様作用が増大する物質と減少する物質があることを指摘し、調製方法や添加塩素濃度に依存して、そのいずれも検出できることを示した[3]。すなわち、XAD-7HP 樹脂を用い水酸化ナトリウムで溶出するという調製方法の場合には、エストロゲン様作用は増大するものとして検出されるが、固相カートリッジOASIS HLB を用いジクロロメタンで溶出するという調製方法をとった場合には、逆に低減する結果となるのである。

以上より、水道原水および水道水の試料濃縮法としては、以下の手順が適当であるとの仮説を設定した。すなわち、

①試料の pH を2に調整する。

②OASIS HLBに通水する。
③水酸化ナトリウムにより溶出する。
④ジクロロメタンにより溶出する。

本法を用いる場合、まずフミン物質が、XAD-7HPと同様にOASIS HLBによって回収できると好都合である。すなわち、フミン酸、フルボ酸の抽出はこれまでXAD樹脂を用いる方法[1]が多用されてきたが、今日では特に微量有機物質の抽出を目的として固相抽出カートリッジと加圧送液システムを用いる方法が普及してきている。そこで、3次元蛍光分析を用いて、OASIS HLBによる濃縮サンプルとXAD-7HPによる濃縮サンプルの比較を行い、フミン物質を中心とする一般有機物の回収性の評価を行うこととした。さらに、いくつかのエストロゲン様作用物質をとりあげその回収性についても検討した。

2. 実験方法

2.1 フミン物質を対象とした濃縮方法

自然水の濃縮法の概略を示す。①琵琶湖南湖表流水を採水。(TOC2.1mg/L) ②0.45μmメンブランフィルターを用いて加圧ろ過。③ろ過水4LをpH2に調整後、コンディショニング済みのXAD-7HP（オルガノ）樹脂カラム（30mm径、95mm長、容積70mL）またはOASIS HLBカートリッジ（Waters）に15mL/mimで通水。④0.1M水酸化ナトリウムで溶出し、XAD-7HPの場合200mL、OASIS HLBの場合10mLを採取。⑤XAD-7HPからの溶出液（200mL）をpH2に調整後、XAD-7HP小カラム（1mm径、8mm長、容積8mL）に再度通水。⑥方法⑤の小カラムを0.1M水酸化ナトリウムで溶出し10mLを採取。⑦方法④で採取したOASIS HLBからの溶出液、および方法⑥で採取した溶出液を、コンディショニング済みの陽イオン交換樹脂（オルガノ IR-120B）に通水し最終的に10mL（400倍濃縮）を得た。

また、琵琶湖水塩素処理水に関しても2Lを同様の濃縮方法で濃縮し最終的に10mL（200倍濃縮）を得た。

得られた溶液を試料として蛍光分析、TOC測定（SHIMADZU TOC-5000A使用）を行った。

2.2 試薬フミン酸を用いた実験

試薬フミン酸溶液（Aldrich, TOC892mg/L）の400倍希釈溶液、およびその塩素処理水を2.1と同様の方法により濃縮した試料を蛍光分析、TOC測定に供した。

2.3 塩素処理方法

処理後の琵琶湖水の残留塩素濃度が約1.0mg·Cl_2/Lとなるように塩素要求量に1.0mgを加えた量の次亜塩素酸ナトリウム溶液を希釈して添加し、密栓、20℃、暗所にて24時間静置した。

2.4 蛍光分析

濃縮した試料を蒸留水で再び希釈した後、石英吸光セルに注入し、分光蛍光光度計（日立製作所、F-4500型）を用いて3次元励起・蛍光スペクトルを測定した。また、ブランクとして蒸留水のスペクトルを測定し、補正を行った。同時にTOC値を測定し、回収率を求めた。

2.5 水道水中のエストロゲン様作用物質を考慮した濃縮方法

著者らはこれまでに、水道原水を対象としたエストロゲン様作用試験のための試料調製法として「pHを2に調整し、OASIS HLBに吸着、ジクロロメタンで溶出する」という方法が適当であることを示した[3]。そこで、ここでは、試料水を以下の方法で濃縮し、TOCおよび個別物質の回収性について検討を行った。①試料水をpH2に調整後コンディショニング済みのOASIS HLBに10mL/minで通水。②0.1M水酸化ナトリウムで溶出し10mLを採取。③水酸化ナトリウム溶出後のカートリッジをジクロロメタンで溶出し、3mLを採取。④窒素吹き付けによりジクロロメタンを乾固後、蒸留水

10mLに再溶解。

水酸化ナトリウム、ジクロロメタンそれぞれによる溶出液を測定試料とし、回収率を測定した。なお、個別物質の濃度測定にはELISA法（武田薬品工業またはIBL）を用いた。

3. 実験結果と考察

3.1 OASIS HLBによる水中フミン物質の抽出性に関する実験結果

塩素未処理水（琵琶湖水）を、XAD-7HP、OASIS HLB両樹脂を用いて、フミン物質を対象とした濃縮方法2.1、すなわち、水酸化ナトリウムによる溶出によって濃縮試料を作製した。試料の蛍光スペクトルを図1に示す。表1にピーク位置、ピーク強度をまとめて示す。これらを比較すると、3ヶ所の蛍光ピークの出現位置が類似していることがわかる。また、これらのピーク1，2の位置は表2に示すフミン物質の蛍光ピーク位置の報告[5]とも類似している。TOC回収率を表3に示す。XAD-7HPによる濃縮で約20%、OASIS HLBによる濃縮で約25%回収している。

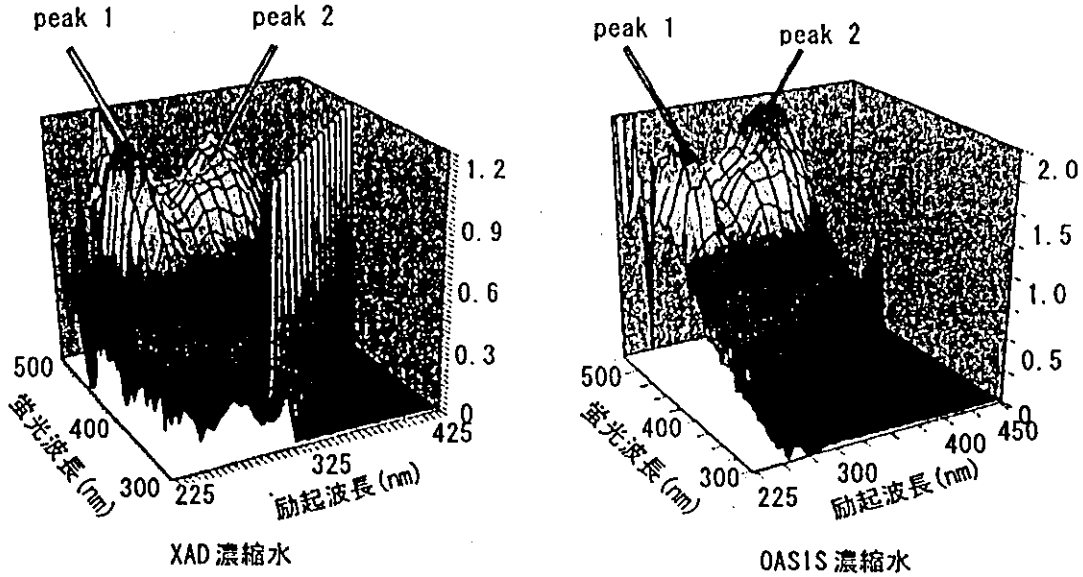

図1 琵琶湖水濃縮サンプルの3次元励起・蛍光スペクトル

表1 琵琶湖水濃縮試料のピーク位置と蛍光強度

		ピーク位置(励起波長/蛍光波長)(nm)	ピーク強度
塩素未処理水	XAD濃縮水	①255/415 ②325/430 ③340/465	①1.22 ②1.08 ③1.04
	OASIS濃縮水	①245/420 ②325/435 ③340/465	①1.95 ②1.91 ③2.02

表2 フミン物質のピーク位置[5]

	ピーク位置(励起波長／蛍光波長)(nm)
フミン物質	①250/435 ②335/435

表3 琵琶湖水サンプルのTOC回収率

		TOC(mg/L)	回収率(%)
琵琶湖水	琵琶湖水(未濃縮)	2.1	――
	XAD濃縮水(400倍)	167	20
	OASIS濃縮水(400倍)	207	25

以上をまとめると、従来のXAD-7HPを用いた濃縮法と比して、OASIS HLBを用いた濃縮法で自然水中のフミン物質を回収できていると判断できる。

塩素処理水について同様な濃縮操作を行い試料を作製した。試料水の蛍光分析の結果を図2に示す。表4にピーク位置、ピーク強度を示す。この場合、XAD-7HPによる濃縮試料で2ヶ所、OASIS HLBによる濃縮試料と未濃縮試料で3ヶ所のピークを検出したが、両樹脂による濃縮試料および未濃縮試料で蛍光ピーク1，2の出現位置が類似している。塩素未処理のものに比べて蛍光波長が低波長側に移動しているが、これは、スペクトルの形状が測定試料中の物質の存在比によって形成されるものであり、また、分子量の小さいグループは、大きいグループと比べて、蛍光ピークの出現波長が低波長側に見られたとする報告[6]があることから、フミン物質と塩素との反応の結果であると考えられる。TOC回収率は両樹脂とも約25%であった。以上の結果から、塩素処理水を対象とした場合にもOASIS HLBを用いて、XAD-7HPを用いた場合と同様の濃縮ができると考えられる。

試薬フミン酸を用いた実験結果を表5に示す。この場合においても、塩素処理前後ともに、XAD-7HP、OASIS HLB両樹脂による濃縮試料および未濃縮試料のスペクトル形状が類似している。また、

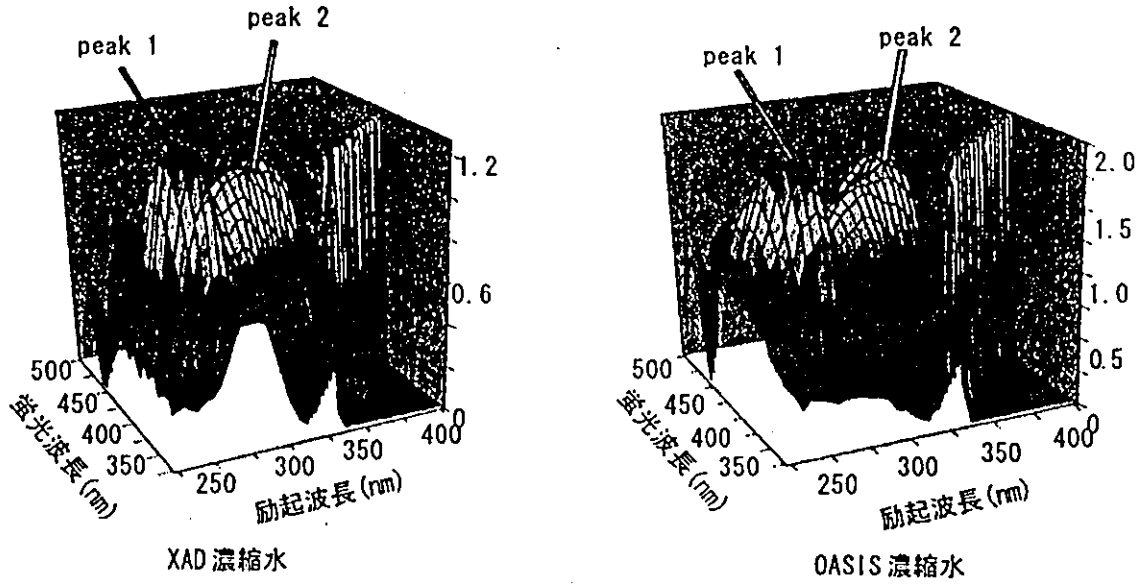

図2 琵琶湖水塩素処理水濃縮サンプルの
3次元励起・蛍光スペクトル

表4 塩素処理水濃縮試料のピーク位置と蛍光強度

		ピーク位置(励起波長/蛍光波長)(nm)	ピーク強度
塩素処理水	塩素処理水(未濃縮)	①265/370 ②325/405 ③345/465	①2.04 ②1.77 ③1.61
	XAD濃縮水	①265/370 ②325/405	①1.31 ②1.11
	OASIS濃縮水	①265/365 ②325/415 ③340/460	①1.95 ②1.75 ③1.59

表5 試薬フミン酸濃縮試料のピーク位置と蛍光強度

			ピーク位置(励起波長/蛍光波長)(nm)	ピーク強度
試薬フミン酸	塩素未処理水	試薬フミン酸希釈水(未濃縮)	①255/480 ②445/525	①0.50 ②0.15
		XAD濃縮水	①255/470 ②445/525	①0.57 ②0.17
		OASIS濃縮水	①255/480 ②445/525	①1.10 ②0.32
	塩素処理水	塩素処理水(未濃縮)	①250/450	①0.30
		XAD濃縮水	①255/445	①0.43
		OASIS濃縮水	①255/470	①0.71

TOC回収率は、XAD-7HIPによる濃縮で約28%、OASIS HLBによる濃縮で約26%となった。よって、水系フミン物質のみではなく、泥炭由来とされるAldrich試薬フミン酸についてもOASIS HLBを用いて回収することができると考えられる。

3.2 水道水中のエストロゲン様作用物質を考慮した濃縮法に関する実験結果

3.1の実験結果を踏まえ、水道水試料の濃縮法として、OASIS HLBを用い、溶出溶媒として水酸化ナトリウムおよびジクロロメタンを併用する濃縮法を用いて、各エストロゲン様作用物質の回収性を検討した結果を図3に示す。TOCの回収性に関しては琵琶湖水ろ過水を、個別物質の回収性に関してはそれぞれの物質を蒸留水中に溶解したものを、それぞれ試料水として2.5の方法に従って濃縮したものである。

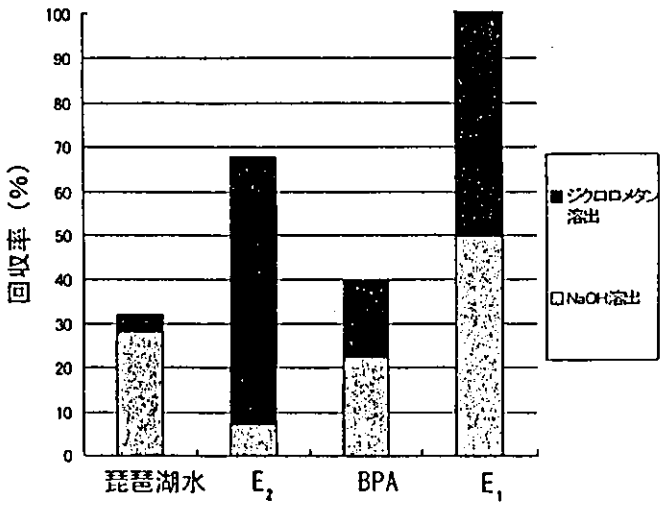

図3 各物質の回収率

琵琶湖水中有機物は、水酸化ナトリウムによる溶出で25%程度回収でき、続くジクロロメタンによる溶出では数%上乗せする程度にとどまった。17β-エストラジオール（E_2）は主に第2段階のジクロロメタンによる溶出で回収され、合計で約70%の回収率となった。ビスフェノールA（BPA）やエストロン（E_1）でも同様に、第2段階のジクロロメタンによる溶出で多く回収できるという結果となった。E_1はELISA特有の過大評価の影響が見られるものの、ジクロロメタンによる溶出が有効であることが確認できる。一方、BPAの場合は回収率の合計は40%にとどまったが、水酸化ナトリウムによる加水分解の影響の可能性も否定できない。

4. 結 言

本文では、水道原水、水道水試料のエストロゲン様作用試験のための方法論を確立することをめざし、一連の実験を行った。得られた結果を以下にまとめる。

(1) 濃縮試料の3次元励起・蛍光スペクトルを比較することにより、OASIS HLB固相抽出カートリッジを用いた濃縮法で、従来のXAD-7HPを用いた濃縮法と同様に水中フミン物質、泥炭由来の試薬フミン酸、およびそれらの被塩素処理物質を濃縮回収できると考えられた。

(2) 水酸化ナトリウムおよびジクロロメタンを用いて2段階で溶出を行う濃縮法により、エストロゲン用作用構成成分を効率よく濃縮できることを示した。

(3) 以上の結果、水道水中のエストロゲン様作用構成成分を考慮した試料濃縮法として、①pHを2に調整。②OASIS HLBに通水。③水酸化ナトリウムで溶出。④ジクロロメタンで溶出。とする方法が適すると考えられる。

水道水のエストロゲン様作用試験のための方法論を確立するにあたっては、濃縮した各物質の相互作用によりエストロゲン様作用強度に影響を及ぼす可能性等も考えられるため、エストロゲン様作用試験を行いつつ検討する必要があり、今後の課題となる。

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CHARACTERIZATION OF ESTROGENIC EFFECTS AND BY-PRODUCTS FORMED DURING WATER DISINFECTION PROCESSES: SOME APPLICATIONS OF MEMBRANE SEPARATION TECHNIQUES

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ABSTRACT

The estrogenic effect of chlorinated Lake Biwa water was up to 2.3 times stronger than that of unchlorinated Lake Biwa water. The main contributor to this elevated estrogenic effect would be chlorination by-products. It should be emphasized that this phenomenon is an analog to the formation of trihalomethanes from natural organic matters. Also, it was found that different molecular size fractions of natural organic matters play different roles on the formation of ozonation by-products. Relatively small molecules in natural organic matters are major precursors of small identifiable ozonation by-products.

INTRODUCTION

The potential risk of endocrine disrupting chemicals (EDCs) in the aquatic environment has been an important issue also in waterworks. Hundreds of compounds are now listed as suspected EDCs. On the other hand, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, 1998) has recommended in the final report that six kinds of mixtures in addition to single compounds be subjected to the assays to evaluate the potential for endocrine disruption. Disinfection by-products (DBPs) in drinking water are included in those mixtures listed.

We consider that it is important to measure estrogenic effects of natural water and by-products by chlorination in addition to commonly suspected EDCs. Therefore, the first part of this paper will discuss the characteristics of estrogenic effects formed by chlorination of drinking water focusing on chlorination by-products.

Ozone is also a good disinfectant especially when the occurrence of *Cryptosporidium parvum* is concerned. However, ozonation is not free from the formation of DBPs. Bromate, organic bromines (*e.g.*, bromoform and bromoacetic acids), and aldehydes are known as ozonation DBPs of health concern (Glaze *et al.*, 1993). While the mechanism of bromate formation during ozonation is well understood (von Gunten and Hoigné, 1994), the role of natural organic matters (NOM) on the formation of organic DBPs is not fully understood. For this reason, the second part of this study will focus on the evaluation of DBPs formed during ozonation of fractionated NOM by membrane separation techniques.

MATERIALS AND METHODS

The MVLN Assay

EDSTAC has recommended the MVLN assay as an estrogen receptor transcriptional activation assay in the screening. The MVLN assay utilizes an MCF-7 derivative that has been stably transfected

with the Vit-Luc reporter gene (Pons *et al.*, 1990). Thus, the MVLN cell line expresses the endogenous estrogen receptor of MCF-7 and at the same time, contains an exogenous estrogen responsive reporter (luciferase). Therefore, the estrogen specific transcription activity of a test chemical is directly related to the activity of luciferase measured in the lysate of treated MVLN cells. In this study, estrogenic effect was measured by the MVLN assay. The MVLN cell line was obtained from its source (Pons *et al.*, 1990). Luciferase activity was detected with a luminometer (Lumat LB9507, Berthold). Concentration of protein in cell lysate was measured using sodium bicinchoninate (Smith *et al.*, 1985), and luminescence units per milligram of protein (relative light units, RLU) were calculated. Relative luciferase activity was calculated as follows using 17β-estradiol as a standard chemical (Itoh *et al.*, 2000).

$$\text{Relative luciferase activity (\%)} = \frac{L_t - L_c}{L_E - L_c} \times 100 \qquad (1)$$

where L_E is RLU of 17β-estradiol at 1.0×10^{-9} M; L_c is RLU of control cell lysate (in 10% DCC FBS media); and L_t is RLU of a test chemical and solution.

Concentration of Lake Biwa Water

Organic matter in Lake Biwa was concentrated by adsorption and desorption method using XAD-7HP (Organo Corporation) resin to isolate and concentrate humic substances. Six litters of Lake Biwa water filtered with a 0.45μm membrane filter of which DOC was 1.8 mg/L was concentrated using XAD-7HP resin according to an isolation procedure of aquatic humic substances (Thurman *et al.*, 1981). The concentration factor was 500 times with a recovery of 12%. As a result, DOC of the concentrate was 110 mg/L.

Filtered Lake Biwa water was chlorinated at an initial concentration of 1.0 mg/L. Residual chlorine and TOX after 24 hours were approximately 0.1 mg/L and 110 μg/L, respectively. Chlorinated water was concentrated by the same procedure for raw water.

Measurement of Molecular Weight Distribution

Molecular weight distribution of TOC of concentrated Lake Biwa water and its change by chlorination was examined. The fractionation was carried out by successive ultrafiltration using three kinds of polycarbonate membrane filters with 14 mm of diameter. YM1, YM10 and YMT (Amicon, Inc.) with 1000, 10000 and 30000 MW cut-off, respectively, were used. 0.8 mL of sample water was added in a MPS micropartition device (Amicon, Inc.) and centrifuged at 1350×g for 30 minutes. TOC of the filtrate was measured.

Fractionation of Raw Waters for Ozonation Experiments

Six municipal water supplies in the Northern Midwest region in the United States were selected as the water sources for ozonation experiments. The three groundwaters selected were the Teays Aquifer (TYS), Urbana, IL, Minot (MIN), ND, and Manhattan (MAN), KS. The three surface waters were Lake Decatur, Decatur (DEC), IL, Lake Vadnais, St. Paul (STP), MN, and the Big Sioux River, Sioux Falls (SFR), SD.

The fractionation of the raw waters was performed using a continuous tangential flow ultrafiltration (UF) and reverse osmosis (RO) membranes. The raw waters were first filtered with a 0.22 μm tangential flow filter (Millipore) to remove particulate matters. Hardness was removed with a Dowex 50x8 resin. The sample was then processed through a continuous flow UF system. The first

step consisted of a 30000 Da polysulfone membrane (Millipore). The retentate (30 K fraction) was continuously recycled through the membrane until there were approximately 2 L of concentrated sample. The permeate was fed to a 1000 Da cellulose acetate membrane (Millipore) to concentrate the molecules between 1000 and 3000 Da (1 K fraction). The permeate from this filter was fed to a Millipore Bench Top Reverse Osmosis unit containing a Millipore Nanomax-95 spiral wound polyamide membrane. The concentrated retentate (RO fraction) contained the material smaller than 1000 Da and larger than the 300-500 Da size exclusion cut-off for the RO membrane. The total recoveries organic carbon varied between 50 and 80%.

Batch Ozonation

Ozonation experiments were conducted in true batch mode. The reactor consisted of a 1-L graduated cylinder with a sliding Teflon disk cover to prevent the volatilization of ozone and DBPs. The reaction time was set to zero minutes when the stock ozone solution was mixed to NOM solutions. A concentrated stock ozone solution was prepared by continuously bubbling ozone gas through distilled water in a gas-washing bottle. This method yielded approximately 30 mg/L of aqueous ozone solution. Ozone was generated from dry ultra high pure oxygen gas by an OREC Model O3V10-0 ozone generator.

Each batch experiment embodied a time series of 1 to 60 minutes for ozone decay evaluation. Other DBP concentrations were measured after approximately 24 hours to allow the evaluation of the ultimate formation potentials. Ozonation experiments were conducted under the following conditions: DOC, 3 mg/L; Br^-, 400 μg/L; pH, 7.5; inorganic carbon, 2 mM; ozone dose, 3 mg/L.[1]

Analytical Procedures

Chlorination

TOX and TOC were measured by a TOX-10Σanalyzer (Mitsubishi Chemical Corporation) and a TOC-5000A analyzer (Shimadzu), respectively. 17β- estradiol (E_2) and 4-nonyl phenol was measured by an enzyme-linked immunosorbent assay (NEOGEN Corporation) and a gas chromatograph (Hewlett Packard 5890 Series II) with a mass spectrometer (JOEL, JMS-AX505), respectively.

Ozonation

A Dohrmann Phoenix 8000 organic carbon analyzer was used to determine the dissolved organic carbon (DOC). Bromate concentration was determined with a Dionex DX-300 ion chromatograph system. Total organic bromine (TOBr) was determined with a Dohrmann Model DX-20A TOX analyzer. The procedure for the determination of aldehydes included the derivatization of aldehydes with *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride followed by extraction with hexane and analysis with a Varian 3700 gas chromatograph (Sclimenti *et al.*, 1990). Concentrations of bromoacetic acids and bromoform were determined by using and slightly modifying USEPA Methods 552.2, 551.1 and 501 (USEPA, 1990; USEPA, 1992; USEPA 1995). Aqueous ozone concentration was determined by the indigo method.

[1] For two raw waters of which DOC values were lower than 3 mg/L, ozonation was conducted with the same ratios of the chemicals to DOC as for other samples. Then, DBP concentrations were normalized on DOC basis (*i.e.*, all concentrations are shown as DBP formation per 3 mg/L of DOC).

RESULTS AND DISCUSSION

Estrogenic effect of Lake Biwa water and chlorinated Lake Biwa water

Table 1 shows quality of raw water and concentrated water with XAD-7HP resin. Recoveries of DOC of Lake Biwa water and the chlorinated one were 12% and 18%, respectively.

Table 1. Quality of Lake Biwa water concentrated with XAD-7HP.

Sample		pH	TOC (mg/L)	E_{260} (10 mm^{-1})	TOX (μg Cl/L)
Lake Biwa water	Raw water	7.6	2.7	0.0398	14.5
	Filtered water	7.4	1.8	0.0191	13.0
	Chlorinated water	7.4	2.5	0.0159	111
Concentrated Lake Biwa water	Filtered water	5.0	107 (11.7)	2.0 (21)	517 (7.1)
	Chlorinated water	3.4	165 (18.2)	2.0 (21)	4980 (9.0)

Table 1 shows quality of raw water and concentrated water with XAD-7HP resin. Recoveries of DOC of Lake Biwa water and the chlorinated one were 12% and 18%, respectively.

Figure 1 shows a result of the MVLN assay of concentrated Lake Biwa water and chlorinated one. The horizontal axis shows the volume of Lake Biwa water added to the media of the MVLN cells. It was found that concentrated Lake Biwa water using XAD-7HP resin has estrogenic effect. It was also confirmed that distilled water prepared by the same concentration procedure does not have estrogenic effect up to 200 mL/mL-media.

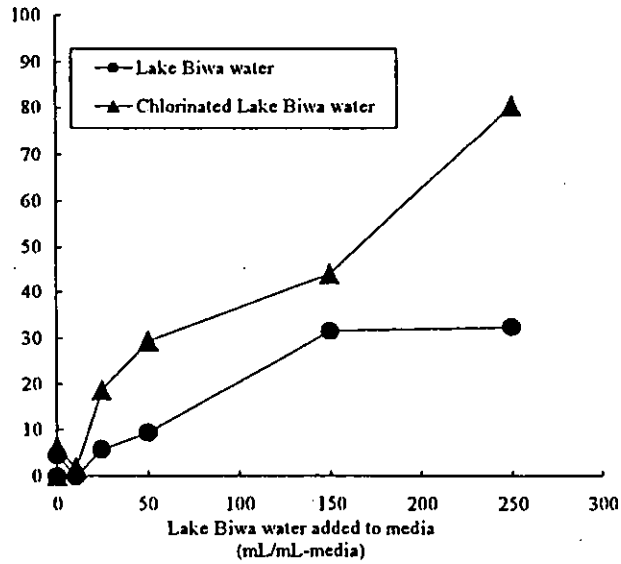

Figure 1. Estrogenic effect of Lake Biwa water concentrated with XAD-7HP.

In addition to this, estrogenic effect increased by chlorination of Lake Biwa water. Estrogenic effect of chlorinated water was approximately 2.3 times as strong as that of Lake Biwa water itself. Itoh *et al.* (2000) has found that commercial humic acid also has the estrogenic effect and it increases by chlorination.

Figure 1 also shows that it is possible to detect estrogenic effect of Lake Biwa water with addition of approximately 50mL/mL-media. This means that sample volume necessary to this assay would be 1.2 L, which is one fifth of 6 L, and required concentration factor would be 100 times.

Factors affecting the increase of estrogenic effect by chlorination

The reasons of increasing estrogenic effect by chlorination could be 1) chlorine produces by-products such as organochlorine substances to be estrogenic ; 2) low-molecular-weight fraction which may bind to the estrogen receptor in a cell increases as a result of oxidation and hydrolysis caused by chlorination ; and 3) chlorine releases estrogenic substances interacting with humic substances in the

aqueous environment (Suffet and MacCarthy, 1989).

Figure 2 shows molecular weight distribution of organic matter in concentrated Lake Biwa water and its change by chlorination. It could be supposed that organic matter less than 1000 of molecular weight can enter a cell. Figure 2 shows the percentage of organic matter less than 1000 increased slightly by chlorination. In contrast, estrogenic effect of chlorinated water was 2.3 times as strong as that of Lake Biwa water itself as shown in Figure 1. Therefore, the effect of chlorination by-products would be larger than that of the increase of low-molecular-weight fraction by chlorination.

Table 2 shows estrogenic effects of some chlorination by-products measured by the MVLN assay. It was found that 2,4-dichlorophenol has weak estrogenic activity. Chlorine would produce estrogenic substances such as chlorophenols as a result of the reaction of chlorine with organic matter in natural water.

In addition to humic substances, individual compounds such as 17β- estradiol and alkyl phenols are present in natural water. Itoh *et al.* (2000) showed that 17β-estradiol (E_2) and 4-nonyl phenol would contribute to the estrogenic effect of Lake Biwa water. Figure 3 and Table 3 show changes of concentrations of those compounds during chlorination and the change of estrogenic effect of E_2, respectively. It is shown that both compounds could be decomposed by chlorine, and the estrogenic effect of E_2 decreased drastically. These compounds are examples showing that the estrogenic effect decreases by chlorination.

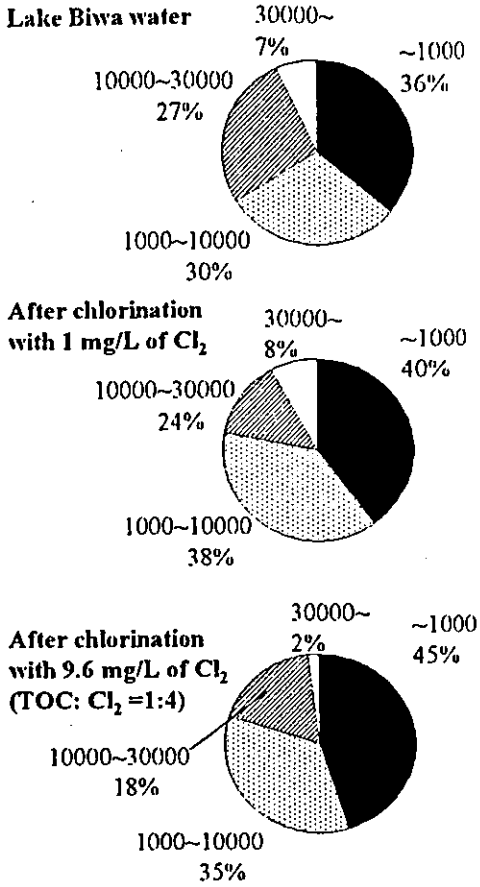

Figure 2. Molecular weight distribution of TOC of concentrated Lake Biwa water and its change by chlorination.

Table 2. Estrogenic effects of chlorination by-products.

Test chemical	Estrogenic activity
Chloroform	0
Dichloroacetic acid	0
Trichloroacetic acid	0
Chloral hydrate	0
o-Chlorophenol	0
2,4-Dichlorophenol	7.7×10^5
17β-Estradiol	1.0

Table 3. Change of estrogenic effect of 17β-estradiol by chlorination.

	Relative luciferase activity (%)
Before chlorination	91
1 day after chlorination	3.3

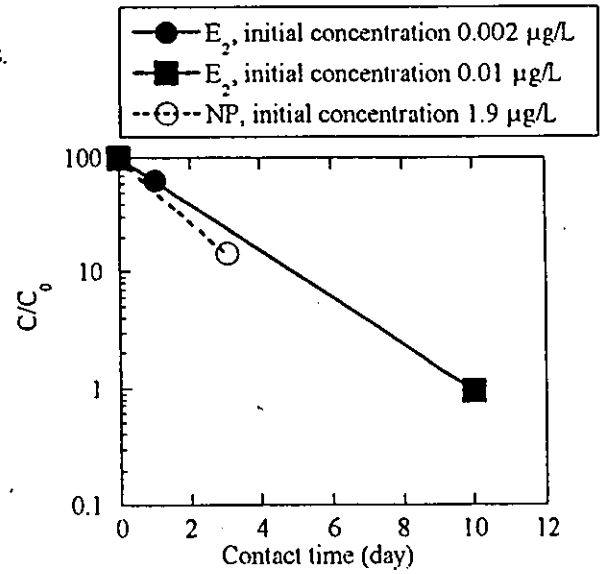

Figure 3. Decomposition of 17β-estradiol and 4-nonyl phenol by chlorine.

These results suggest that estrogenic substances would be decomposed by residual chlorine, when chlorine releases estrogenic substances interacting with humic substances in the aqueous environment.

The obtained results suggest that the main factor affecting the increase of estrogenic effect by chlorination would be the effect of chlorination by-products.

This also shows that the estrogenic effect is formed as a result of the reaction of chlorine with organic matter in natural water. The important point is that this finding (elevated estrogenic effects by chlorination) is very similar to the production of trihalomethanes from natural waters.

DBP formation from ozonation of fractionated NOM

Figure 4 shows the comparison of bromate formation between different fractions (RO, 1K, and 30 K) and raw waters. RO fractions and raw waters showed higher bromate formation than 1K and 30 K fractions. Also, ozone decay rates were much faster in 1 K and 30 K fractions than in raw waters and RO fractions *i.e.*, ozone exposures were smaller for 1 K and 30 K fractions (Figure 5). One major reason for the faster ozone decay in large molecular size fractions may be higher unsaturated carbon contents in these fractions. Unsaturated compounds, such as aromatic compounds, are known to react with ozone faster than other saturated compounds. Specific UV absorbance (SUVA), an indicator of unsaturated carbon contents, tended to be higher for larger molecular size fractions.

The average TOBr levels of the six raw water waters 17 μg/L, and the average TOBr levels of their 30 K, 1 K, and RO fractions were 8, 14, and 12 μg/L, respectively (Figure 6). The higher TOBr levels in raw waters were attributed to the slower ozone decay. The low TOBr formation from the RO fraction despite the slower ozone decay than those of 1 K and 30 K fractions, could be due to higher hydrophilicity of the brominated compounds since the activated carbon for TOBr analysis concentrates only relatively hydrophobic compounds and the DBPs from RO fractions are considered more hydrophilic (*e.g.*, more carboxylic compounds) than those in higher molecular size fractions.

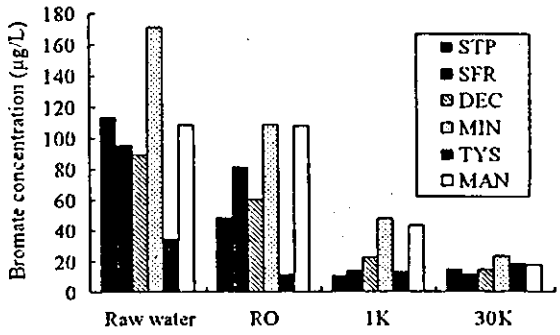

Figure 4. Comparison of bromate formation between different molecular size fractions.

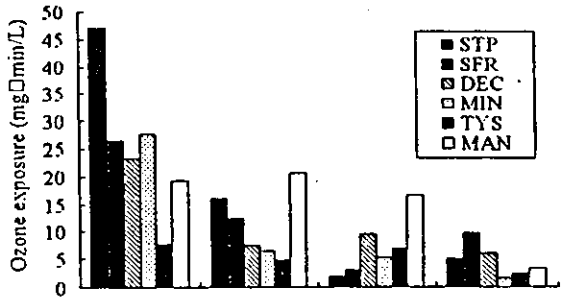

Figure 5. Comparison of ozone exposure between different molecular size fractions.

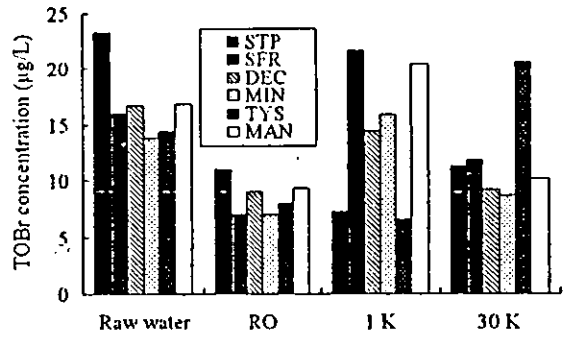

Figure 6. Comparison of TOBr formation between different molecular size fractions.

More bromoform was formed in the raw waters than other fractions (Figure 7). Especially, bromoform levels in the RO fractions were below detection limit (0.15 μg/L) for the six sources. 1 K and 30 K fraction also showed very low bromoform formation except for the 1 K fraction of the MAN sample. It is of note that even with relatively high ozone exposure, bromoform was not formed in the RO fractions. This indicates that the RO fraction contains fewer carbonyl groups that are susceptible to undergo the haloform reaction than other fractions (and probably more carboxylic groups).

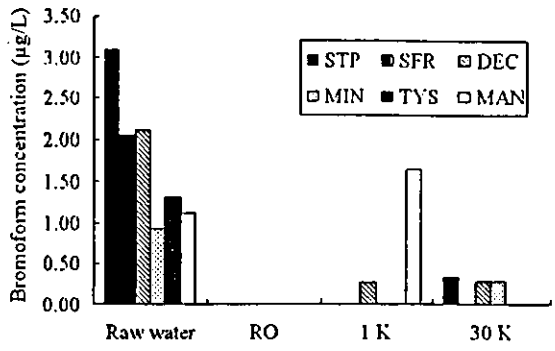

Figure 7. Comparison of bromoform formation between different molecular size fractions.

Interestingly, raw waters produced more bromoacetic acids than fractionated isolates (Figure 8). This tendency was similar to those for bromoform, but more distinct for bromoacetic acids. With similar TOBr levels of 1K and 30K fractions to that of raw waters and lower individual organic bromine concentrations in larger molecular size fractions, it was implied that TOBr formed from larger molecular size fractions are larger and more complicated organic bromines than bromoform and bromoacetic acids.

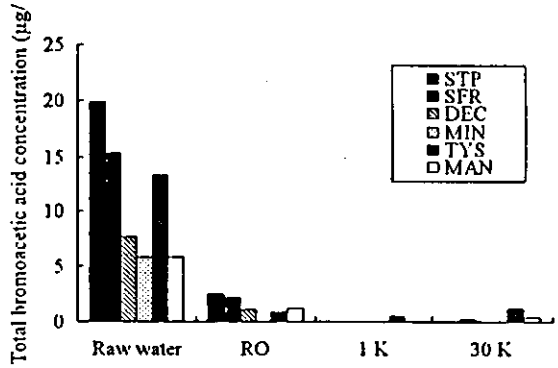

Figure 8. Comparison of bromoacetic acid formation between different molecular size fractions.

Figure 9 shows the comparison of total aldehyde levels between the different molecular size fractions. The RO fraction yielded the highest aldehyde level, and the total aldehyde levels of 1 K and 30 K fractions were lower than those of the raw waters. The higher aldehyde levels of the raw water and the RO fractions were considered to be caused by two main reasons: 1) higher ozone exposure (*i.e.*, more ozone) is available in the presence; 2) larger aldehydes that are supposed to be produced from larger molecular size fractions are not detectable with the method. Also, it is of note that the percentages of glyoxal and methyl glyoxal in 1K and 30 K fractions were higher than those in the raw water and the RO fractions (data not shown).

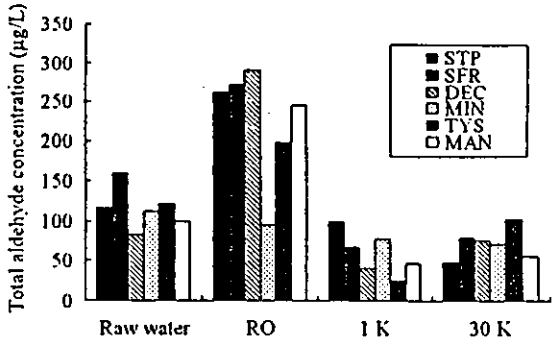

Figure 9. Comparison of aldehyde formation between different molecular size fractions.

In short, small identifiable ozonation DBPs appear to be formed mainly from relatively small molecules in NOM mixtures. On the other hand, larger molecules seem to rapidly consume ozone to form large uncharacterized DBPs.

CONCLUSIONS

The estrogenic effect of Lake Biwa water increased by chlorination up to 2.3 times as strong as that of unchlorinated Lake Biwa water. The main factor affecting the increase of estrogenic effects by chlorination would be the formation of chlorination by-products. It should be emphasized that this phenomenon is very similar to the formation of trihalomethanes in drinking water treatment process in that NOM are major precursors of both estrogenic effect and trihalomethanes. Also, it was found that different molecular size fractions of NOM play different roles on the formation of ozonation DBPs. It is of note that small molecules in natural organic matters are major precursors of small identifiable ozonation DBPs such as bromoacetic acids and small aldehydes.

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